



Evaluation of Antioxidant Enzymatic Activity, DNA Damage, and Quality of Fresh Semen in Kundhi Buffalo Bulls

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ABSTRACT

This investigation aimed to assess semen quality, antioxidant enzymatic activity, lipid peroxidation, and sperm DNA integrity within fresh semen samples obtained from Kundhi buffalo bulls. The fresh semen samples from individual bulls were evaluated for parameters including volume, pH, mass motility, individual sperm motility, viability, morphology, and sperm concentration. Then semen were pooled and reevaluated aforementioned quality parameters. In addition membrane integrity, antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), via malondialdehyde (MDA) concentration and sperm DNA fragmentation was evaluated. Statistically significant differences ($P < 0.05$) were identified among individual bulls concerning semen volume, pH, sperm motility, viability, morphology, and sperm concentration. One bull (B) notably exhibited comparatively superior seminal characteristics. Analysis of the pooled fresh semen revealed mean values of 6.73 ± 0.024 for pH, $85.38 \pm 0.46\%$ for sperm motility, $93.13 \pm 0.39\%$ for viability, $94.13 \pm 0.39\%$ for normal morphology, and 1132 ± 10.66 million/mL for sperm concentration. The activities of the antioxidant enzymes CAT, SOD, and GSH were determined to be 28.13 ± 0.35 U/mL, 96.63 ± 0.37 U/mL, and 80.38 ± 0.32 μ M, respectively. Correspondingly, the MDA concentration was 2.50 ± 0.26 nmol/mL, and sperm DNA damage amounted to $3.13 \pm 0.29\%$. These findings collectively suggest a favorable seminal quality, characterized by low oxidative stress and minimal DNA damage, in the fresh semen of Kundhi buffalo bulls.

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Authors' Contribution

KAM carried out the experimental work, gathered and analyzed the data and drafted the manuscript. NAK, study design, supervision of study, statistical analysis. HST, AK data evaluation, manuscript writing, and review. AK data evaluation and manuscript review. AAM provided financial support.

Key words

Kundhi Buffalo bull, Fresh semen, Antioxidant enzymes, Sperm DNA damage, Oxidative stress

INTRODUCTION

Buffalo populations constitute a substantial component of the livestock economy across numerous Asian nations, notably in Pakistan, where they provide significant contributions to milk production, meat supply, and the sustenance of rural livelihoods (Anas *et al.*, 2023). Among indigenous breeds, the Kundhi buffalo is recognized as a significant dairy type, primarily due to its

demonstrated adaptability to challenging environmental conditions and its inherent capacity for milk yield. The genetic enhancement of this breed relies considerably on the utilization of superior breeding bulls within artificial insemination programs, given that semen quality represents a primary determinant of reproductive efficiency and fertility. Consequently, a comprehensive evaluation of semen characteristics in breeding bulls becomes imperative for advancing reproductive performance and safeguarding valuable germplasm (Shao *et al.*, 2021).

Spermatozoa are highly specialized cells requiring structural and functional integrity for successful fertilization. Conventional parameters for semen evaluation, encompassing sperm motility, viability, morphology, concentration, and membrane integrity, are widely employed as indicators of semen quality and fertilizing potential (Neglia *et al.*, 2020). However, these conventional assessments alone frequently prove insufficient for accurately predicting fertility, as sperm function is additionally influenced by biochemical and

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molecular factors, particularly oxidative stress and DNA integrity. Recent investigations have underscored that assessing oxidative status and sperm DNA damage provides a more dependable estimation of semen quality and reproductive competence in breeding bulls (Sharafi *et al.*, 2022).

Reactive oxygen species (ROS) are continuously generated during normal cellular metabolism and fulfill a physiological role in processes such as sperm capacitation, hyper activation, the acrosome reaction, and fertilization (Aitken, 2017; Arain *et al.*, 2023). Nevertheless, an excessive production of ROS disrupts the oxidant–antioxidant balance, leading to oxidative stress, which detrimentally affects sperm function (Benko *et al.*, 2022). Spermatozoa are particularly susceptible to oxidative damage due to the high concentrations of polyunsaturated fatty acids within their plasma membrane and the limited antioxidant defense mechanisms present in their cytoplasm (Brito, 2025). Excessive ROS production can induce lipid peroxidation, mitochondrial dysfunction, membrane destabilization, diminished motility, apoptosis, and DNA fragmentation, ultimately compromising fertilizing ability (Neglia *et al.*, 2020).

To mitigate oxidative stress, seminal plasma incorporates an elaborate antioxidant defense system. This system comprises enzymatic antioxidants, such as superoxide dismutase (SOD) (Sharafi *et al.*, 2022), catalase (CAT) (Kowalczyk, 2022), and glutathione peroxidase (GPx), alongside various non-enzymatic antioxidants. These antioxidant enzymes act to neutralize ROS and shield spermatozoa from oxidative injury (Pei *et al.*, 2023). Variations in antioxidant enzymatic activity have been correlated with differences in semen quality and fertility among breeding males. A reduction in antioxidant defense mechanisms may heighten the susceptibility of spermatozoa to oxidative damage, thereby impairing sperm motility, viability, and membrane integrity. Consequently, evaluating antioxidant enzymatic activity has emerged as an important method for understanding the physiological status and fertilizing potential of semen (Sharafi *et al.*, 2022).

Among the various ramifications of oxidative stress, sperm DNA damage has garnered considerable attention, primarily because an intact chromatin structure is indispensable for successful fertilization and normal embryonic development. DNA fragmentation in spermatozoa may arise from oxidative stress, defective chromatin packaging, apoptosis, or various environmental and physiological stressors. Elevated levels of DNA damage have been linked to reduced fertility, impaired embryo development, and early embryonic loss (Sharafi *et al.*, 2022). Previous studies conducted on buffalo bulls

have demonstrated a close relationship between sperm DNA integrity and semen quality traits, as well as fertility potential. Mahmoud *et al.* (2015) reported a significant inverse correlation between DNA damage and sperm motility in buffalo semen, suggesting that chromatin integrity assessment could serve as a complementary indicator of semen quality.

Buffalo spermatozoa are considered more vulnerable to oxidative damage compared with those of other livestock species, primarily due to their elevated membrane content of polyunsaturated fatty acids (Brito, 2025). While several studies have explored oxidative stress and sperm quality in frozen buffalo semen (Iqbal *et al.*, 2018), there remains limited information regarding antioxidant enzymatic activity and DNA integrity in the fresh semen of Kundhi buffalo bulls. The evaluation of fresh semen is particularly important as it reflects the intrinsic reproductive health and physiological status of breeding bulls prior to semen processing and cryopreservation. Gaining a comprehensive understanding of the relationship among antioxidant enzyme activity, DNA damage, and semen quality parameters may facilitate the identification of biomarkers for selecting superior breeding bulls and ultimately enhance reproductive efficiency in buffalo breeding programs.

Therefore, the current investigation was designed to evaluate antioxidant enzymatic activity, DNA damage, and semen quality characteristics in fresh semen obtained from Kundhi buffalo bulls. The findings derived from this study may contribute to a more profound understanding of the biochemical and molecular determinants influencing semen quality and provide valuable insights for refining semen evaluation protocols and reproductive management strategies within buffalo breeding initiatives.

MATERIALS AND METHODS

Selection of animals

This study was conducted at the Semen Production Unit of the Department of Animal Reproduction, Sindh Agriculture University, Tandojam. Four mature buffalo bulls (A, B, C, D) of similar age and body conditions were selected as semen donors. The animals were housed individually and managed. Animals were fed 5 kg green grass, 1.5 kg wheat straw, 0.5 kg concentrate and 50 gm mineral mixture per 100 kg body weight per day with *ad libitum* water.

Semen collection and evaluation

Semen was collected using an artificial vagina at a maintained temperature at 42 °C. Two conservative's ejaculates were collected from each kundhi bull twice

weekly for a period of four weeks (replicates). Immediately after semen collection, they were transferred to laboratory and kept in a water bath at 37 °C. Each ejaculate was determined for volume, color, pH, motility, sperm motility, morphology, viability, and sperm concentration. Sperm motility was determined by a phase-contrast microscope at 200x magnification. Sperm concentration was determined using a hemacytometer for the method described by [Yang et al. \(2016\)](#). The selected ejaculates were pooled. After pooling, the semen was reevaluated for volume, pH, mass, motility, morphology, viability, and motility. In addition, sperm DNA damage and antioxidant activities (catalase, superoxide dismutase, and glutathione peroxidase) were assessed in pooled fresh semen samples.

Volume, pH of semen and mass activity

Glass graduated collection tubes were used to measure and record semen volume in milliliters ([Goswami et al., 2020](#)). Semen pH was measured by using Digital pH meters (Eutech). Mass activity was performed by placing 15µl fresh semen on prewarmed (37°C) slide and assessing without a cover slip under low magnification at 10X using phase contrast microscope. The grading of mass activity was according to the wave motion pattern, (grade 0: Waves are absent, spermatozoa nonmotile, grade 1: Waves are absent, spermatozoa motile, grade 2: Scantly differentiable waves in motion, grade 3: Waves patent, moderate motion, grade 4: Dark, clear waves in fast motion) ([Malak et al., 2026](#)).

Sperm motility

A drop of diluted semen (normal saline 1:100 ratio) was sited on preheated slide (37 °C), covered by a covered slip. Sperm motility was observed under phase-contrast microscope (40 ×) ([Iqbal et al., 2018](#)). For motility, 100 spermatozoa were counted randomly, and results were expressed as a motility percentage.

Morphology and viability of sperm

Sperm viability and morphology were determined using the eosin nigrosine stain. 10µL of dilute sample was mixed with two to three drops of eosin nigrosine stain on a prewarmed glass slide. After 3 min incubation at 37 °C, another slide was used to create a thin smear. The smear was air dried and observed under phase-contrast microscope (40 ×). Sperm without stain were considered viable, whereas stained spermatozoa were considered non-viable ([Yang et al., 2016](#)).

Concentration of sperm

Sperm concentration was determined using hemocytometer. The fixing solution was prepared with

30ml of sodium chloride (3%), 4ml of formaldehyde (37%), and 1000ml of distilled water. Briefly, 0.01 ml of semen was diluted with 9.99 ml of fixing solution and allowed to stand for five min. A drop of diluted semen was placed onto each of the hemocytometer's chambers and observed under a phase-contrast microscope at 40X ([Iqbal et al., 2018](#)). Spermatozoa were counted in 5 squares (1 central square, 4 corner squares). The number of sperm was calculated using the formula below.

$$\text{Sperm cell/ml} = N \times 5 \times DF \times 10 \times 1000.$$

Where; N = number of sperm counted. DF= diluted factor (100).

Antioxidant enzyme analysis

The antioxidant enzyme, including superoxide dismutase(SOD), catalase (CAT) and total glutathione (GSH), were evaluated in fresh semen samples (120 µl) were centrifuged at 1600 x g for 15 min to separate seminal plasma. The resulting pellet was treated with 360µl of 1% Triton X-100 and incubated for 20 min. Subsequently, the samples were centrifuged at 4000 x g for 30 min at 25 °C, and supernatant containing the enzyme extract was collected. Enzymatic activity was determined using commercial assay kits: catalase (OxiSelect™ STA-339, Cell Biolabs, USA), superoxide dismutase (Sigma-Aldrich 19160) and total glutathione (OxiSelect™ STA-312, Cell Biolabs, USA) ([Partyka et al., 2012](#)).

Lipid peroxidation (Malondialdehyde)

Lipid peroxidation was determined by measuring malondialdehyde (MDA) levels using thiobarbituric acid assay. Semen samples containing 40 x 10⁶ spermatozoa were mixed with sodium dodecyl sulfate solutions, acetic acid (pH 3.5), and thiobarbituric acid. The mixture was heated at 95 °C for 60 min, then allowed to cool at room temperature. After the addition of a butanol pyridine solution (15:1), the samples were centrifuged at 4000 x g for 10 min. The absorbance of the supernatant was measured using an ELISA reader (HER-480, HT company Ltd, UK) at 492 nm. MDA concentrations were calculated from the standard curve prepared using tetramethoxypropane ([Banday et al., 2017](#)).

Evaluation of sperm DNA damage

Sperm DNA fragmentation was evaluated in fresh semen using the Halomax DNA fragmentation kit. Semen samples with a final concentration of 15-20x 10⁶ sperm/mL were diluted in phosphate buffer saline. Agarose was melted at 90-100 °C and cooled at 37 °C, then mixed with semen samples. The mixture was spread onto slides and incubated at 4 °C for five min. Slides were treated with lysis solution, washed, and dehydrated in graded ethanol

solutions (70%, 90%, and 100%) for 4 min each, followed by air drying. Slides were stained sequentially with eosin and methylene blue and examined under a microscope at 400 x magnification, using a green filter. DNA damage was assessed based on halo formation around the sperm head. At least 500 spermatozoa were evaluated per sample.

Statistical analysis

Data were analyzed using one way ANOVA in SPSS software (version 24.0, IBM, USA). Mean difference among bulls were compared using Tureky post hoc test. Results are presented as mean $\pm$ SE and significance was considered at $P < 0.05$.

RESULTS

Characteristics of fresh semen

Semen volume exhibited significant differences ($P < 0.05$) across the bulls, with bull B recording the highest ejaculate volume at 4.90 ml (Table I). This was followed by bull A (4.80 ml), bull D (4.66 ml), and bull C (4.50 ml). Similarly, semen pH (Table I) also varied significantly ($P < 0.05$). Bull B displayed the highest pH value (6.90), whereas bull C registered the lowest at 6.65.

Table I. Mean value of volume, pH, mass motility, motility, morphology, viability, sperm concentration, of four Kundhi buffalo bulls semen.

Parameters	Bull A	Bull B	Bull C	Bull D
Volume (ml)	4.80 $\pm$ 0.08 ^{ab}	4.90 $\pm$ 0.07 ^a	4.50 $\pm$ 0.09 ^c	4.66 $\pm$ 0.08 ^{bc}
pH	6.70 $\pm$ 0.03 ^b	6.90 $\pm$ 0.04 ^a	6.6500 $\pm$ 0.03 ^b	6.68 $\pm$ 0.03 ^b
Mass motility	4.00 $\pm$ 0.00 ^a	4.00 $\pm$ 0.00 ^a	4.00 $\pm$ 0.00 ^a	4.00 $\pm$ 0.00 ^a
Motility (%)	85.750 $\pm$ 0.85 ^{ab}	86.875 $\pm$ 0.78 ^a	84.000 $\pm$ 0.92 ^b	85.375 $\pm$ 0.81 ^{ab}
Morphology (%)	95.125 $\pm$ 0.98 ^a	96.000 $\pm$ 0.91 ^a	93.250 $\pm$ 0.85 ^b	92.375 $\pm$ 0.78 ^b
Viability (%)	94.375 $\pm$ 0.78 ^a	94.875 $\pm$ 0.88 ^a	92.125 $\pm$ 0.97 ^b	91.125 $\pm$ 0.88 ^b
Concentration (Million/ml)	1134.4 $\pm$ 20.2 ^b	1200.0 $\pm$ 18.6 ^a	1103.1 $\pm$ 16.4 ^b	1090.6 $\pm$ 19.3 ^b

Conversely, mass motility (Table I) remained consistent among all bulls, each achieving a score of 4.00, which suggests no significant difference ($P < 0.05$). In contrast, individual sperm motility showed significant variation ($P < 0.05$), with bull B demonstrating the highest motility percentage (86.88%). Bull C, however, exhibited

the lowest motility at 84.00% (Table I).

Significant differences ($P < 0.05$) were also observed in both sperm morphology and viability percentages (Table I). Bulls A and B displayed higher percentages of normal sperm morphology (95.13% and 96.00%, respectively) when compared to bulls C and D (93.25% and 92.38%, respectively). A similar pattern was noted for sperm viability, with bulls A and B showing greater viability (94.38% and 94.88%, respectively), while bulls C and D recorded lower viability percentages (92.13% and 91.13%, respectively).

Sperm concentration also differed significantly ($P < 0.05$) among the bulls (Table I). Bull B achieved the highest sperm concentration, measured at 1200.0 million/ml, while bull D had the lowest concentration, recorded at 1090.6 million/ml. Collectively, bull B generally demonstrated a more favorable profile for several fresh semen quality characteristics among the Kundhi buffalo bulls evaluated.

Table II. Mean value of pH, mass motility, motility, morphology, viability, sperm concentration, catalase, superoxide dismutase, glutathione, malondialdehyde, and sperm DNA damage in fresh pooled semen of Kundhi buffalo bulls.

Fresh semen parameters	Mean $\pm$ SE (n=8)
pH (1-7)	6.73 $\pm$ 0.024
Mass motility (1-5)	4.00 $\pm$ 0.22
Motility (%)	85.38 $\pm$ 0.92
Morphology (%)	94.13 $\pm$ 0.79
Viability (%)	93.13 $\pm$ 0.87
Sperm concentration (million/ml)	1132 $\pm$ 10.66
Catalase (U/mL)	28.13 $\pm$ 0.35
Superoxide dismutase (U/mL)	96.63 $\pm$ 0.37
Glutathione (μ M)	80.38 $\pm$ 0.32
Malondialdehyde (nmol ml ⁻¹)	2.5 $\pm$ 0.26
Sperm DNA damage %	3.125 $\pm$ 0.29

Characteristics of fresh pooled semen

Table II presents the average values of seminal pH, Mass motility, Sperm concentration, CAT, SOD, GSH, MDA and Sperm DNA Damage observed in fresh pooled semen collected from Kundhi buffalo bulls. The ejaculate pH 6.73 $\pm$ 0.024. The mean mass motility score was 4.12 $\pm$ 0.22, while individual sperm motility was recorded as 85.38 $\pm$ 0.46%. The mean sperm viability and normal morphology were 93.13 $\pm$ 0.39% and 94.13 $\pm$ 0.39%, respectively. The sperm concentration was observed 1132 $\pm$ 10.66 million/ml. With respect to antioxidant

status, the activities of CAT, SOD and GPx were 28.13 ± 0.35 U/mL, 96.63 ± 0.37 U/mL, and 80.38 ± 0.32 μ m, respectively. The mean level of MDA was 2.50 ± 0.26 nmol/mL, while the percentage of sperm DNA damage was 3.13 ± 0.29 .

DISCUSSION

The present study showed significant bull-to-bull variation in fresh semen quality of Kundhi buffalo bulls. Bull B exhibited the most favorable semen profile, with the highest semen volume, pH, individual motility, normal sperm morphology, viability, and sperm concentration, whereas bulls C and D showed comparatively lower morphology, viability, and concentration. In contrast, mass motility remained similar among all bulls, indicating that mass motility alone may not be sufficient for accurate semen-quality assessment.

These findings are partly similar to [Kaka et al. \(2016\)](#), who evaluated Kundhi buffalo bull semen and reported variation mainly in sperm morphology and membrane integrity, while mass activity and several other conventional semen parameters showed non-significant differences. In the present study, significant differences were observed in a wider range of parameters, including volume, pH, individual motility, morphology, viability, and concentration. This difference may be related to bull age, season, nutrition, management, collection interval, or semen-evaluation methods.

The higher concentration and motility observed in bull B agree with [Kumar et al. \(2023\)](#), who reported clear variation in ejaculate volume, sperm concentration, mass activity, initial motility, post-thaw motility, total sperm number, and motile sperm number among Murrah buffalo bulls. Similarly, [Amin et al. \(2024\)](#) reported that semen traits such as volume, mass motility, live sperm percentage, abnormal sperm percentage, and concentration are important indicators for evaluating Egyptian buffalo bulls. These studies support the present finding that multiple semen parameters should be considered when selecting breeding bulls.

The sperm concentration of bull B (1200.0 million/ml) is also supported by [Bansal et al. \(2025\)](#) who reported that buffalo ejaculates within a suitable concentration range can contribute effectively to semen-dose production and cryopreservation. Therefore, bull B may have better potential for semen processing and artificial insemination. However, concentration should not be interpreted alone; it should be assessed together with motility, morphology, viability, and post-thaw semen quality.

The higher sperm viability and normal morphology in bulls A and B may indicate better spermatogenesis,

epididymal maturation, and sperm membrane stability. In contrast, the lower morphology and viability observed in bulls C and D may reflect reduced sperm structural integrity or greater cellular stress. [Bhave et al. \(2020\)](#) also reported that buffalo semen quality is influenced by breed, bull effect, age, collection interval, season, and management conditions. Furthermore, [Fataniya et al. \(2025\)](#) showed that sperm motility, viability, acrosome integrity, mitochondrial function, and DNA fragmentation are associated with fertility differences among buffalo bulls, supporting the need for advanced semen-quality testing in future studies.

The present study provides baseline results of the seminal characteristics and antioxidant activity of fresh pooled semen from Kundhi buffalo bulls. The semen volume (4.71 ± 0.04 mL) is slightly higher than previously reported values but remains within the typical range reported for buffalo (1.3-8.0 mL). Ejaculate volume is influenced by several factors, such as breed, age, season, and management ([Iqbal et al., 2018](#)). The mean semen pH in present study (6.73 ± 0.024) indicated a near neutral to slightly acidic environment, which is consistent with the normal physiological pH range reported for buffalo semen (6.5-7.0). Maintenance of an optimal pH environment is important for sperm metabolism, motility, and overall fertilizing ability ([Brito, 2025](#)). Mass motility and individual motility are widely used as indicators of semen quality. The mean mass motility score (4.12 ± 0.22) and individual sperm motility (85.38 ± 0.46 %) observed in the present study indicate excellent quality of Kundhi bull semen. Similar motility values have been reported in other breeds such as Murrah and Nili Ravi. High sperm motility generally reflects intact plasma membranes and functional mitochondria, both of which are essential for fertilization ([Shah et al., 2017](#)). Similarly, the high sperm viability (93.13 ± 0.39 %) and normal morphology (94.13 ± 0.39 %) observed in this study, further confirm the good quality of the collected semen samples. The findings are consistent with a previous study in buffalo bulls ([Kaka et al., 2016](#)). A high proportion of viable and morphologically normal spermatozoa is strongly associated with improved fertilization potential, as structural abnormalities and reduced viability can reduce the capacity of spermatozoa to reach and successfully fertilize the oocyte ([Munari et al., 2019](#)).

The sperm concentration recorded ($1,132 \pm 10.66$ million/mL) also falls within range reported for buffalo bulls. Adequate sperm concentration is essential to ensure enough viable spermatozoa per insemination dose. Thereby enhancing the probability of successful fertilization ([Castro et al., 2025](#)).

CONCLUSION

The overall, semen quality was excellent, antioxidant activity in fresh semen was good, lipid peroxidation was low and minimum sperm DNA damage was observed in fresh semen of Kundhi buffalo bulls and hence good reproductive potential of the buffalo bulls were observed. The results indicate that in addition to the materials of semen analysis, assessment of antioxidant enzymes and sperm DNA integrity can be useful to select the better quality breeding bulls to improve the reproductive efficiency of buffalo breeding programs.

DECLARATIONS

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This study was re-viewed and ap-proved by the Directorate of Advanced Studies and Research (DASAR), Sindh Agriculture University, Tandojam, Sindh, Pakistan.

Ethical statement

All proce-dures in-volving animals were con-ducted in accord-ance with the institutional guidelines for the care and use of animals in research and were approved by the Directorate of Advanced Studies and Research (DASAR), Sindh Agriculture University, Tandojam, Sindh, Pakistan.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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